

EXHIBIT 21

PART 2

of human serum: 1.35 ml. of the stock suspension are pipetted into a test tube, centrifuged and the supernatant fluid discarded. One ml. of human serum is added to the sediment taking care to obtain a smooth suspension. The mixture is incubated at 37°C. for one hour with occasional mixing to keep the zymosan in suspension. Centrifuge, pour off the inactivated serum and dilute with 4 ml. of saline to obtain an approximate 1/5 dilution with respect to original serum volume.

Guinea pig serum requires about fifteen times as much zymosan for inactivation of C'3 (100) as human serum. The optimal quantity of zymosan for human as well as guinea pig serum should be determined by experiment, since different preparations of zymosan may vary in potency. It may also be necessary to allow one and one-half or two hours instead of one hour of incubation. Choice of the amount of reagent and time of inactivation should be based on actual tests for completeness of C'3 inactivation with minimal destruction of the other components. McNall (114) suggests inactivation of guinea pig C'3 with PZ made from human serum (cf. properdin section).

C. Destruction of C'4: To 1 ml. of serum is added 0.25 ml. of 0.15 N NH₄OH. Incubate for one and one-half hours and neutralize with 0.25 ml. of 0.15 N HCl. Add saline to a final dilution of 1/5 with respect to original serum volume. Alternatively, hydrazine can be used at one-half the molar concentration needed for ammonia.

D. Heat Inactivated Serum: Human and guinea pig sera are heated at 56°C. ± 0.1° for twenty minutes. This is sufficient to inactivate C'1 and C'2. There is also partial loss of C'3 and C'4. Add saline to a dilution of 1/5.

Titration

The titers of individual components are estimated by dilution as in an ordinary

complement titration except that a constant amount of reagent (R1, R2, R3 or R4) for the component in question is added to all the tubes. The amount chosen must be inactive when tested with sensitized sheep cells, and it must not be anti-complementary. Anticomplementary properties are ascertained by testing the effect of the reagent on 1/2, 1/4, 1 and 1 1/2 100% units of whole C'. If one unit, or even 1 1/2 units, fail to lyse, the reagent is anti-complementary and should be used at a lower concentration. In the case of R1 and R4 an enhancing effect is usually observed since these reagents supply components which are present in low titer in human and guinea pig sera. R2 is the reagent which most commonly exhibits anticomplementary effects especially when the midpiece, which is part of this reagent, is old and has been allowed to become too alkaline (cf. studies by Lepow *et al.*, refs. 115-117).

The sequence of tests in a typical experiment is summarized below:

1. Test M, E, Z, N and H alone for complete inactivation, using from 0.05 to 0.4 ml. of a 1/5 dilution in two-fold steps.
2. Check the anticomplementary properties of M, E, Z, N and H.

Use not more than 1/2 the least anticomplementary dose of a reagent for the subsequent tests.

Tests No. 1 and No. 2 thus define the *upper limits* for the use of each reagent.

3. Titrate H against Z. The following is a sample protocol;

ml 1/5 or 1/10 dilution of Z	ml 1/5 or 1/10 dilution of H		
	0.05	0.10	0.20
0.05			
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This titration should determine the lower limits of activity of H and Z.

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A similar series of titrations of H against N is done next, but since the titer of C'4 in H is quite high, a dilution of 1/20 or 1/40 of H should be used.

These tests will show how much H should be used in reagents R1 and R2. It has been found best to keep E and H, and M and H apart and to add them separately to the tests requiring R1 and R2.

4. A series of titrations of Z against N, using Z at a dilution of 1/10 and N at a dilution of 1/15, as in 3 above, should yield information regarding the *lower limits* for Z and N as reagents R3 and R4, respectively.

5. M and E are titrated against one another in the presence of the optimal amount of H, as determined in test No. 3. M can usually be run at a dilution of 1/20 while E is ordinarily used at 1/10. This

experiment should furnish the *lower limits* for M and E.

The dilutions indicated here are given for general guidance but may require upward or downward revision depending on the results of the titrations.

The tests, thus far, serve to establish the composition of R1 (E + H) with respect to its content of C'2, C'3 and C'4, as well as that of R2 (M + H) with respect to C'1, C'3, and C'2. It remains to be determined whether Z and N contain sufficient C'1 and C'2 to serve as R3 and R4, respectively. This is done by testing Z and N with R1 and R2, which are reagents for C'1 and C'2 respectively. These reagents are then used in amounts which furnish enough of the desired components and which are not anticomplementary or lytic. If any reagent does not fulfill these requirements, it should be discarded.

SEQUENCE OF ACTION OF COMPLEMENT COMPONENTS AND ISOLATION OF INTERMEDIATE PRODUCTS

From experiments with the reagents R1, R2, R3 and R4 Pillemer *et al.* (118), as well as Bier and Trapp (119), deduced that the components of complement act sequentially in the order C'1, 4, 2, 3 or C'4, 1, 2, 3. Definitive information on the nature of the successive reaction steps and the properties of the intermediate products emerged from more recent studies on the role of Ca^{++} and Mg^{++} , and on the kinetics of the hemolytic reaction.

Resolution of the Ca^{++} and Mg^{++} Steps

As noted before, the hemolytic activity of guinea pig complement is enhanced by Mg^{++} . In 1951 Levine *et al.* studied the role of Ca^{++} and Mg^{++} in the uptake of complement nitrogen by specific precipitates (120). The experiments, which were made following the technique described earlier, showed clearly that EDTA interferes with the uptake of complement nitrogen by antigen-antibody aggregates, just as it blocks hemolysis by complement.

In a typical experiment, shown in Table 7, a specific precipitate picked up 0.042 mg. N from 2.0 ml. of fresh, undiluted guinea pig serum as a source of complement. Concomitantly, hemolytic activity disappeared from the fluid phase. By contrast, in the presence of EDTA, only 0.014 mg. N was fixed by the specific precipitate, and there was little loss of hemolytic activity from the fluid phase (measured after restoration of Ca^{++} and Mg^{++}). Furthermore, addition of Ca^{++} and Mg^{++} served to overcome the inhibitory effect of EDTA on the fixation of complement.

The uptake of 0.014 mg. of N in the presence of EDTA poses a puzzling problem. The possibility cannot be ruled out that this material has nothing to do with complement, but represents some other nitrogenous substance which becomes incorporated in the specific precipitate.

Maurer and Weigle (121) have confirmed that EDTA inhibits uptake of complement nitrogen from guinea pig and human serum.

TABLE 7

EDTA-inhibition of C' N Uptake by Immune Precipitates at 26° C, and its Reversal by Divalent Cations

Tube	A	B	C	D	E	F	G	H	I	J
C', ml.	2.0	2.0	—	—	—	—	2.0	2.0	2.0	2.0
iC', ml.	—	—	2.0	2.0	—	2.0	—	—	—	—
Anti-Pn III, ml.	0.5	—	0.5	—	0.5	0.5	0.5	0.5	0.5	0.5
EDTA, 0.04 M, ml.	—	—	—	—	—	—	—	0.5	—	0.5
CaCl ₂ , 0.05 M, each, ml.	—	—	—	—	—	—	—	—	0.5	0.5
MgSO ₄ , ml.	—	0.5	—	0.5	0.5	0.5	0.5	0.5	0.5	0.5
S III, ml.	1.5	1.5	1.5	1.5	3.0	1.0	1.0	1.0	0.5	—
Veronal buffer, ml.	0	.002	.002	.008	.630	.650	.692	.654	.666	.676
N precipitated, mg*	0	.002	.002	.008	.632	.654	.680	.664	.684	.690
					.634	.650	.690	.660	.702	
Mean N precipitated, mg.		.002		.008	.632	.651	.687	.659	.684	.683
Blank to be subtracted, mg.					.008	.002	.002	.002	.002	.002
Specific N precipitated, mg.					.643	.685	.685	.657	.682	.681
iC' values to be subtracted, mg.						.643	.643	.643	.643	.643
C'N precipitated, mg.						.042	.042	.014	.039	.038
Total C'N in supernate	395	388					<5	356**	<5	<5

*Corrected for reagent blank

**Optimal quantities of Ca⁺⁺ and Mg⁺⁺ were added to the supernates immediately prior to the titration for hemolytic activity.

Temperature, 26° C.; Reaction time, 90 minutes; diluent, veronal buffer; Rabbit anti-pneumococcus Type III serum No. 796 diluted 2-fold; Pneumococcus polysaccharide, S III, 0.15 mg. per ml.; EDTA, 0.04 M; divalent cation solution, containing 0.05 M Ca⁺⁺ and 0.05 M Mg⁺⁺. Guinea pig serum used as C'. Contents of the tubes were thoroughly mixed every ten minutes. (From ref. 120).

On the other hand, in the case of rabbit serum, they found that EDTA did not block fixation of complement, as measured by uptake of nitrogen. However, the question may be posed whether all of the co-precipitating nitrogenous material should be considered as complement, in line with the definition of Muir (16), as "that labile substance which is taken up by antigen-antibody aggregates." The inadequacy of this definition has been pointed out.

The role of Ca⁺⁺ and Mg⁺⁺ in C' fixation has been studied further, in a dilute system yielding no visible precipitate, by the quantitative method of complement fixation in which loss of hemolytic activity is measured rather than uptake of complement nitrogen (122-128). The method, which will be described in a later section, involves interaction of approximately 100 "50%" units of complement with an antigen-antibody system, followed by measurement of the residual hemolytic activity. From this, and from appropriate control measurements, the number of units of

complement, which have been fixed, can be calculated. If performed under carefully controlled conditions, this procedure yields results with a precision of about $\pm 5\%$, affording a sensitive and reliable tool for investigation of exacting problems, such as the question at issue here.

There are two ways in which a distinction between the functions of Ca⁺⁺ and Mg⁺⁺ can be achieved. One approach rests on the use of a "depleted" reaction system, i.e., a system from which Ca⁺⁺ and Mg⁺⁺ have been removed by treatment of all constituents with an ion exchange resin. By addition of either Ca⁺⁺, or Mg⁺⁺, or of other cations, to the depleted system, their distinct functions can be studied.

The other approach involves the use of the Ba⁺⁺ or Mg⁺⁺ complex of EDTA as an inhibitory agent, in place of free EDTA. Since EDTA binds Ca⁺⁺ more firmly than Ba⁺⁺ or Mg⁺⁺, the addition of the Ba⁺⁺ or Mg⁺⁺ complex of EDTA to a complement-fixation system results in the binding

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Complement and Complement Fixation

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of Ca⁺⁺ by way of displacement of Ba⁺⁺ or Mg⁺⁺ from its complex with EDTA.

Both approaches have yielded the same information, viz., that Ca⁺⁺ plays an essential role in complement fixation, and that its function is specific in the sense that other cations cannot be substituted (75). For example, in a complement fixation experiment with guinea pig complement treated by ion exchange resin, as shown in Table 8, 105 units of complement were fixed in the presence of 0.00015 M Ca⁺⁺, and 100 were fixed in the presence of both Ca⁺⁺ and Mg⁺⁺. In the absence of either divalent cation (i.e., none added) 58 units were fixed, and in the presence of Mg⁺⁺, 47 units were fixed. Thus, it is evident that Ca⁺⁺ potentiates the fixation of complement, while Mg⁺⁺ appears to be without effect; if anything, there is a slight inhibitory action of Mg⁺⁺. However, since the depleted system may still contain a trace of Ca⁺⁺, it is not clear whether this cation is essential.

This question has been answered in experiments with Ba-EDTA as chelating agent. Results, given in Figure 47, showed

TABLE 8
Potentiation of C' Fixation by Ca⁺⁺*

Set	Final Concentration of cation added	C'H ₅₀ fixed
A	Mg ⁺⁺ , 0.0005 M	47
B	Ca ⁺⁺ , 0.00015 M	105
C	Ca ⁺⁺ , 0.00015 M	100
D	Mg ⁺⁺ , 0.0005 M	58

*2 μ g Rabbit-anti-human serum albumin nitrogen + resin treated C' + 0.5 μ g human serum albumin nitrogen; Reaction volume = 10.0 ml. Fixation for 20 hours at 2-4°C; Lysis for 60 minutes at 37°C. in the presence of optimal Ca⁺⁺ and Mg⁺⁺.

that addition of Ca⁺⁺ overcomes the inhibitory effect of Ba-EDTA on complement fixation in a stoichiometric fashion, i.e., 5 micromoles of Ca⁺⁺ just sufficed to neutralize inhibition by 5 micromoles of EDTA. Mg⁺⁺ or Sr⁺⁺ were without effect. Consequently, Ca⁺⁺ is essential (75).

Since complement fixation is an integral part of the immune hemolytic mechanism, it is reasonable to deduce that Ca⁺⁺ is also required for the hemolytic action of complement, in addition to the need for

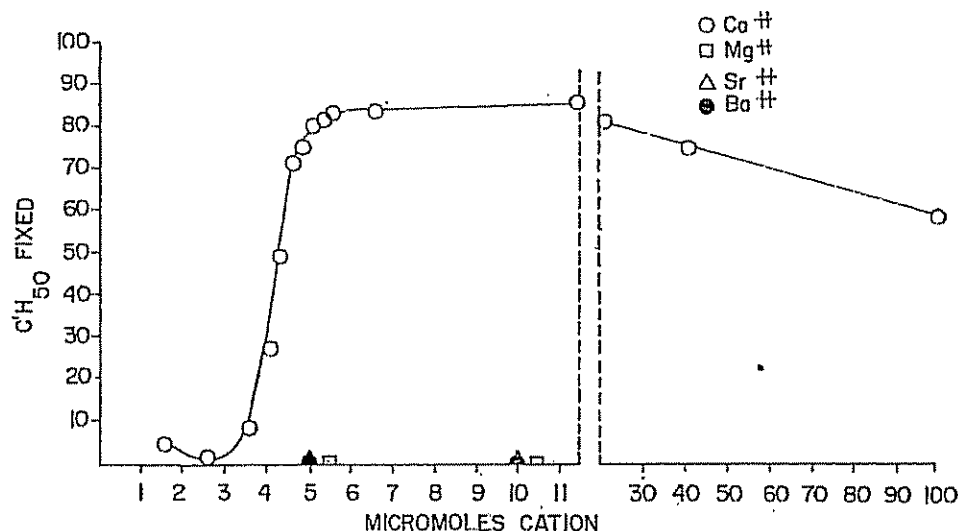


FIG. 47. Addition of five micromoles of Ca⁺⁺ reverses inhibition of complement fixation by five micromoles of Ba⁺⁺-EDTA. Mg⁺⁺ or Sr⁺⁺ are without effect (from ref. 75).

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Mg^{++} . Experimental demonstration of the Ca^{++} requirement in hemolysis has been achieved with complement depleted of Ca^{++} and Mg^{++} by means of ion exchange resin. Although more effective than dialysis, this treatment does not abolish hemolytic activity, but merely reduces the titer. In the best experiment of this type a reduction of 88% in activity has been obtained, i.e., an eight-fold differential of activity between a depleted system and a system to which Ca^{++} and Mg^{++} were restored. In assessing the role of Ca^{++} it is, therefore, necessary to choose a dilution of depleted complement, which does not cause lysis, unless Ca^{++} and Mg^{++} are added. For example, a typical experiment, shown in Figure 48, indicated that with both cations added in optimal concentrations, approximately 30% lysis was achieved. Neither Mg^{++} nor Ca^{++} alone served to restore activity to the resin-treated C' , but both cations were required. Furthermore, it was observed that the requirement for Ca^{++} is highly specific in the sense that Co^{++} , Ba^{++} , Zn^{++} , Mn^{++} , Cd^{++} , Ni^{++} or Mg^{++} , could not be substituted for Ca^{++} . On the other hand, Co^{++} or Ni^{++} can be

substituted for Mg^{++} , but their efficiency is lower (129).

Additional evidence on the significance of Ca^{++} in immune hemolysis has come from studies by Cowan (130) on the mechanism of hemolysis by complement and Carbowax 4000, a polyethylene glycol. It has been recognized for a long time that sensitization of erythrocytes to the action of complement can be accomplished with certain non-specific agents, such as tannic acid or silicic acid. In a follow-up study of McVickar's observation (71) that Carbowax enhances the hemolytic action of complement on red cells sensitized with hemolytic antibody, Cowan discovered that lysis could be produced by the action of Carbowax and complement in the absence of hemolytic antibody, indicating that Carbowax is a non-specific sensitizing agent. Similar observations were made with dextran and polyvinylpyrrolidone. Studies on the mechanism of action showed that Carbowax probably combines with certain constituents of the red blood cell surface, but the union is quite weak and appreciable hemolytic action can be achieved only at relatively high concen-

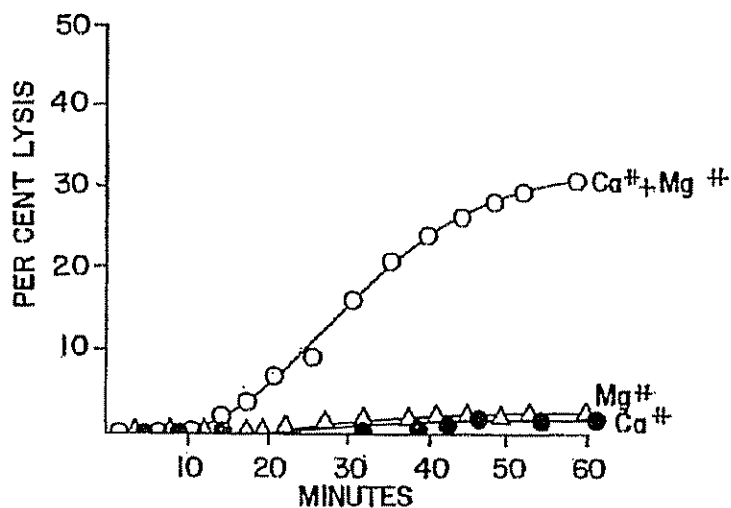


FIG. 48. Potentiation of immune hemolysis by addition of Ca^{++} and Mg^{++} to a reaction system depleted of divalent cations by treatment with ion exchange resin (from ref. 129).

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tration of Carbowax, e.g., 2 to 6%. It was also found that all components of complement are required, suggesting that the mechanism is similar to, or identical with, that operating in immune hemolysis, i.e., that Carbowax merely substitutes for antibody in initiating the series of reactions mediated by the components of complement. However, optimal efficiency of the Carbowax-complement system required a concentration of $0.00075\text{ }M\text{ }Ca^{++}$, which is five times greater than that needed in the antibody-guinea pig complement system. Thus, the enhancing effect of Ca^{++} , which is barely discernible with antibody and guinea pig complement, unless the reaction system is depleted of Ca^{++} by the use of Mg^{++} -EDTA, or by treatment with ion exchange resins, becomes readily apparent in the Carbowax-complement system, as normally constituted.

From these observations it is clear that the hemolytic action of complement involves at least two reaction steps, one requiring Ca^{++} , the other necessitating Mg^{++} . By kinetic experiments (129) it has been demonstrated that the step requiring Ca^{++} precedes that in which Mg^{++}

functions. Thus, as shown in Figure 49, in a reaction mixture containing both cations at the beginning of an experiment, i.e., at the moment of addition of complement to sensitized erythrocytes, hemolysis did not commence until about ten minutes later. If, on the other hand, Ca^{++} was present at zero-time and Mg^{++} was added about twenty-three minutes later, hemolysis began almost immediately following addition of Mg^{++} . Conversely, if Mg^{++} was present at zero-time and Ca^{++} was added at about twenty-three minutes, the lag period was not eliminated. Indeed, it was somewhat greater than in the case in which both cations were present at zero-time. These observations indicate that the reaction between sensitized erythrocytes and certain components of complement in the presence of Ca^{++} requires an appreciable period of time, and that the reaction step involving Mg^{++} cannot get under way until the preceding Ca^{++} step has taken place.

There has been some disagreement from Pillemer's group (131) about the essential nature of Ca^{++} because treatments, such as dialysis or passage through ion exchange resin, for removal of these cations have

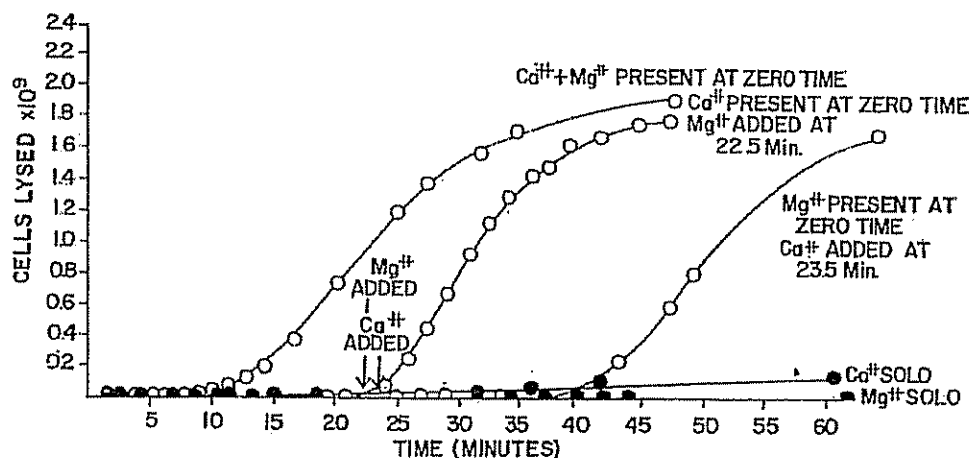


FIG. 49 In a kinetic experiment with guinea pig complement as limiting factor the lag period was about 10 minutes if both Ca^{++} and Mg^{++} were present at zero-time. If Ca^{++} was present at zero-time and Mg^{++} was added 22.5 minutes after introduction of complement, there was almost no lag following addition of Mg^{++} . If Mg^{++} was present at zero-time, and Ca^{++} was added 23.5 minutes after introduction of complement, there was a lag of about fifteen minutes following addition of Ca^{++} . These observations indicate that the Ca^{++} precedes the Mg^{++} step (from ref. 129).

not yielded completely inactive systems. Only direct addition of chelating agents, such as citrate, pyrophosphate or EDTA to the hemolytic system produces complete inhibition. While Lepow *et al.* (131), have confirmed the potentiation of complement fixation by addition of Ca^{++} to a depleted system, they feel that this is a sparing effect on $\text{C}'1$. Furthermore, they believe that interference by the chelating agents is due to intrinsic inhibitory action and not solely and entirely to binding of Ca^{++} and Mg^{++} .

While it is true that free EDTA causes some destruction of complement, this effect is overcome by chelation of EDTA with Ba^{++} or Mg^{++} . Yet, the Ba^{++} -EDTA complex inhibits complement fixation completely, and only Ca^{++} , of all the divalent cations tried, will overcome the inhibition. Therefore, the requirement for Ca^{++} is believed to be absolute and specific, at least in the case of guinea pig complement. In support of this view, the experiments of Laporte (79) and Becker (80) show that Ca^{++} functions as a ligand in the fixation of $\text{C}'1$.

It appears likely that the depletion treatments have not yielded completely inactive hemolytic systems because some of the serum Ca^{++} may be bound quite firmly to serum proteins. Furthermore, the lytic reaction is extremely sensitive to traces of Ca^{++} and Mg^{++} . For this reason, in experiments with a depleted system, all the glassware must be rinsed immediately before use with buffer which has been depleted of Ca^{++} and Mg^{++} by passage through an ion exchange resin. There is an appreciable contribution of divalent cations from pipettes, test tubes or flasks, as evidenced by enhanced hemolytic action when the rinsing treatment is omitted.

From their studies on Ca^{++} and Mg^{++} (120, 75, 129), Levine *et al.* constructed a hypothesis that in the presence of Ca^{++} , sensitized erythrocytes, EA, react with certain components of complement, provisionally designated $\text{C}'x$, to form a com-

plex $\text{EAC}'x$. If Mg^{++} is present, this complex then reacts with other components of complement, provisionally designated $\text{C}'y$, to form $\text{EAC}'x\text{C}'y$. This hypothesis has been tested and found acceptable in studies on the isolation of the intermediate product $\text{EAC}'x$, by preparation of $\text{C}'y$ as a separate reagent, and by investigation of the interaction of $\text{EAC}'x$ with $\text{C}'y$.

Isolation of $\text{EAC}'x$ has been accomplished by treatment of EA at 0°C . with undiluted complement in the presence of Ca^{++} , but absence of Mg^{++} (132). After ten minutes' reaction, the mixture is centrifuged, and after removal of the fluid phase, the cells, which are in the state $\text{EAC}'x$, are washed and suspended in buffered diluent. The intermediate product $\text{EAC}'x$ is stable on washing, and retains full activity on storage either at 0° or 37°C ., provided Ca^{++} is present.*

Similarly, in a reaction system containing Ca^{++} , but lacking Mg^{++} , treatment of guinea pig serum with a specific precipitate (or stromata-antibody complex) removes all detectable $\text{C}'x$, and leaves most of the $\text{C}'y$ in the fluid phase, yielding a reagent, designated $\text{C}'y$, which hemolyzes $\text{EAC}'x$ in the presence of Mg^{++} (132). Sensitized cells, i.e., EA, are not hemolyzed by $\text{C}'y$, even at high concentration of this reagent.

It should be pointed out that the ability to prepare the intermediate complex $\text{EAC}'x$, as well as the reagent $\text{C}'y$, rests on the fact that the union between EA and guinea pig $\text{C}'x$ is firm, i.e., there is little or no dissociation tendency. In contrast, Hoffmann has noted that with Cynomolgus monkey complement the union between $\text{C}'x$ and EA, or other antigen-antibody complexes, appears to possess appreciable dissociation tendency, and as a result it has not been possible to prepare satisfactory monkey $\text{C}'y$, or to make the complex of EA with monkey $\text{C}'x$.

*Guinea pig serum contains a factor which destroys $\text{EAC}'x$; hence, this intermediate product is stable only if serum has been removed by washing.

Complement and Complement Fixation

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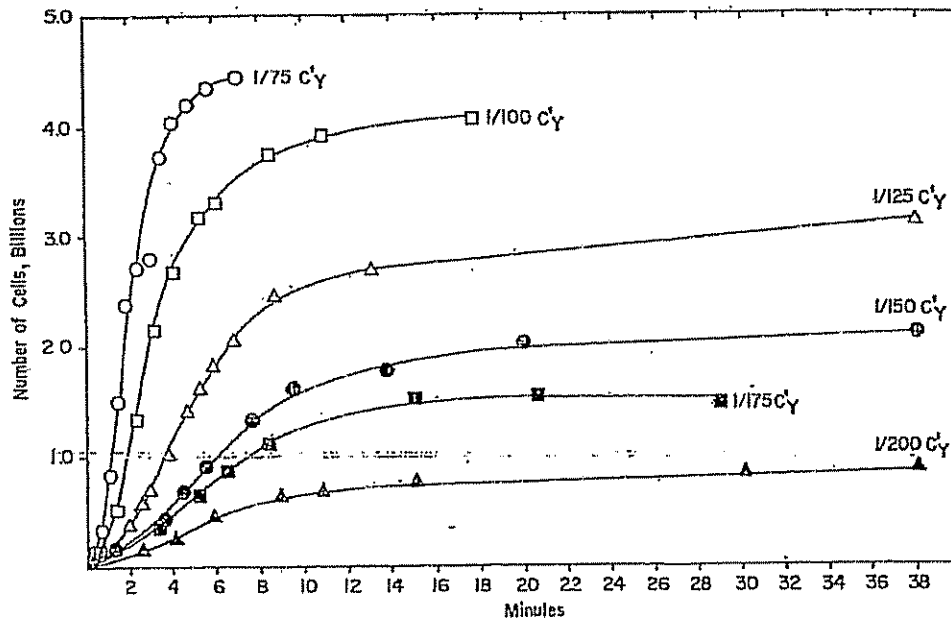


FIG. 50. The effect of concentration of C'y (C'2,3) on its reaction with EAC'x (EAC'1,4). From (132.)

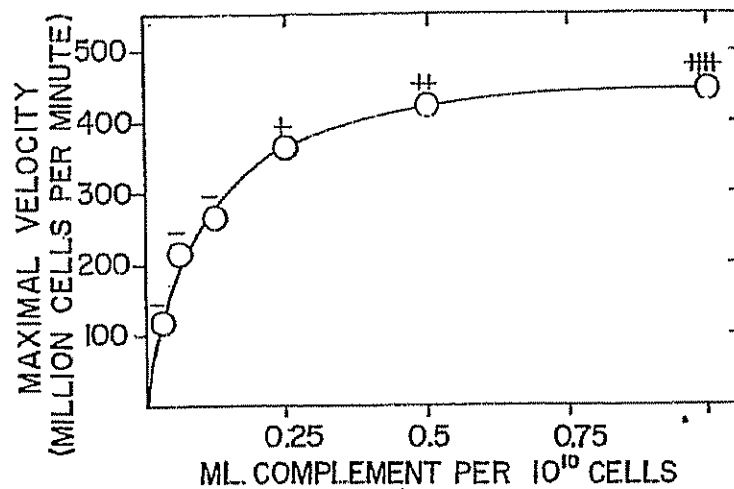


FIG. 51. Maximal velocity of reaction between EAC'x (EAC'1,4) and C'y (C'2,3) as a function of the amount of complement used for the preparation of EAC'x. (Presence of C'x in the supernatant fluid from each of the EAC'x preparations is indicated by +, ++, or +++; Absence of C'x in the supernate by -). (From 132).

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TABLE 9
Qualitative Tests for Complement Components

Substrate	Reagent				
	R1	R2	R3	R4	Heated C'
EAC' _x	++++	0	0	++++	0
EA + C'y (1/10)	±	++++	++++	±	0
EA	0	0	0	0	0

0 = no hemolysis; + = 25% hemolysis; ++ = 50% hemolysis; +++ = 75% hemolysis; ++++ = 100% hemolysis.
From (23).

A typical experiment with guinea pig EAC'_x and C'y is shown in Figure 50. The reaction curves are sigmoidal, but the lag periods are very short. In this reaction only Mg⁺⁺ is required. From experiments of this type some of the properties of the C'x and C'y reaction steps were elucidated. For example, as shown in Figure 51, the reactivity of EAC'_x with C'y depends on the amount of complement used in the preparation of EAC'_x. Thus, each point on the graph represents a different population of EAC'_x with respect to the amount of complement used in its preparation. It is evident that on increasing the quantity of complement in the preparation of EAC'_x, the reactivity of this complex with C'y rises until an optimum is reached with a preparatory dose of approximately 1 ml. of undiluted complement per 10¹⁰ cells (sensitized with about 10¹⁰ molecules of antibody). It is also of interest to note that with small doses of complement all of the C'x is removed by EA, leaving only C'y in the fluid phase. However, with larger amounts of complement, the capacity of EA for the uptake of C'x is apparently exceeded, and free C'x is left in the supernate. As might be expected, it has been found that the capacity of EA to take up C'x depends on the amount of antibody employed in the sensitization of the cells, and this is the reason why increase of antibody enhances the lytic effect of a given quantity of complement, unless the point is reached where C'x becomes exhausted.

From studies of the reaction between EAC'_x and C'y it was also learned that the concentration dependence of the hemolytic activity of complement (56) operates in the C'y step. By contrast, the C'x reaction displays hardly any concentration effect, at least in the case of guinea pig complement. Similarly, it was found that the C'y reaction is highly sensitive to temperature change, i.e., on cooling to 0°C. it is greatly retarded, a factor of significance with respect to sampling technic in kinetic experiments.

From studies with the classical reagents, R1, R2, R3 and R4 for the identification of the components of complement, it has been found, as summarized in Table 9, that C'x comprises C'1 and C'4, while C'y includes C'2 and C'3 (133). Hence, the symbol EAC'_x can be replaced by EAC'1, 4 and this intermediate product is lysed by action of C'2 and C'3.

Action of C'1

It has been shown by Laporte *et al.* (79) and Becker (80) that C'1 can be eluted from cells in the state EAC'1, 4 or EAC'1, 4, 2 by treatment with EDTA.* The eluted C'1 will combine with EA, if Ca⁺⁺ is restored, to form EAC'1. After washing, this product can be hemolyzed by R1, as a source of

*Treatment of EAC'1, 4 with EDTA results in loss of reactivity with C'2, 3. On the other hand, EAC'1, 4, 2 treated with EDTA will react with C'3 and undergo lysis.

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C'2, C'3 and C'4. These observations indicate that C'1 and C'4 react with EA in that order. The eluate containing C'1 also exhibits TAME (p-toluenesulfonyl-L-arginine methyl ester) esterase activity and Becker (80) showed that on treatment of EA with the eluate both C'1 and esterase activity combined with the cells. He has advanced the hypothesis that the C'1 molecule contains two sites, a binding site and an enzymatic site, and that both of these are required for manifestation of C'1 activity. Becker has also presented experiments showing that cells in the state EAC'1, 4 or EAC'1, 4, 2 hydrolyze TAME (134).

This line of investigation is related to studies by Pillemer's group (135, 131, 136) on the possible relationships between complement fixation, destruction of complement by plasmin formed from plasminogen upon addition of streptokinase, and the participation of C'1 and Ca^{++} in these reactions.

It was suggested that antigen-antibody complexes, or plasmin, convert C'1 from a precursor form to an active enzyme which destroys C'2 and C'4. According to these investigators, Ca^{++} promotes the destruction of C'2 and C'4 by activated C'1.

Studies on the possible esterase function of C'1 were stimulated by the observation of Levine (137) that di-isopropyl fluorophosphate (DFP) inhibits the hemolytic activity of complement, presumably by action on the intermediate product EAC'1, 4. Becker also studied the inhibitory effect of DFP and concluded that the precursor form of C'1 in serum is resistant to DFP (138).

The intermediate EAC'1 can be prepared by treatment of EA with R4, but it is better to use a midpiece made from this reagent. It seems that guinea pig serum contains a destructive factor which is removed on separation of the midpiece from R4 (79, 139). According to Klein, treatment of guinea pig complement with ether destroys this destructive agent, as

well as C'4, and, hence, ether-treated complement can be used for preparation of EAC'1 (139). Alternatively, Hoffmann has prepared EAC'1 with a fraction of guinea pig serum made by chromatography on DEAE-cellulose (140).

Lepow *et al.* (115-117) have made detailed studies of partially purified C'1 with respect to the loss of hemolytic activity as tested with R1, and the appearance of esterase activity (tested on N-acetyl-L-tyrosine ethyl ester), which occur in such preparations during storage at pH 7-8 and ionic strength less than 0.15 (117). At 10°C. both processes require several hours, while at 37°C. these changes occur in a few minutes. At pH 6, or lower, and at ionic strength 0.3, or in the presence of EDTA, these changes do not take place. Under a wide range of physico-chemical conditions a positive correlation was found between the rate of disappearance of C'1 and the rate of esterase activation (117), suggesting a similarity to the activation of trypsinogen to trypsin. The C'1 preparations used in this work were free of detectable C'2 and C'4, but they contained C'3, "properdin," plasminogen, as well as the recently described clot-promoting fractions, Christmas factor and Hageman factor (141). It was of interest that addition of commercial streptokinase (Varidase) led to destruction of C'1 activity and activation of esterase at ionic strength 0.3, presumably through activation of the plasminogen present as impurity.

Thus, the studies of Pillemer, Lepow and Becker appear to point in the same direction, but there are some open questions, notably with respect to inactivation of hemolytic C'1 activity by antigen-antibody aggregates. The work of Laporte *et al.* (79) and Becker (80) shows that hemolytically active C'1 can be dissociated from an antigen-antibody complex, while Pillemer and Lepow claim inactivation. Pillemer suggested that Ca^{++} functions as an activator, while Becker and Laporte consider it a ligand.

With respect to the action of streptokinase on complement the criticism may be raised that the product which was used is a crude material derived from streptococcal culture filtrate which contains a variety of antigenic components. The partially purified C'1, or the human serum serving as a source of complement, may contain some of the corresponding antibody, and hence, complement fixation could occur during activation of the plasmin by streptokinase.

Becker has presented much evidence on the bifunctional character of C'1 but the possibility cannot be ruled out that the esterase might be a distinct molecule which has the capacity to combine with antigen-antibody complexes containing C'1. By analogy, it has been noted that beef serum conglutinin acts in this way (18). Furthermore, saline eluates of antigen-antibody-complement complexes, which Lepow prepared, contained Hageman factor and streptokinase-activable plasminogen in addition to esterase (142). The possibility must still be considered that the presence of esterase in antigen-antibody-complement complexes, and its activation,

might be a side effect rather than a direct and essential part of the cytotoxic action of the complement system.

Resolution of the Action of C'y into the Constituent C'2 and C'3 Reaction Steps

As shown in Figures 52 and 53 the limited antibody and the limited complement systems differ in respect to their susceptibility to interference by EDTA. The fact that EDTA arrests the hemolytic process in the limited complement system only if added during the first few minutes, (Fig. 53) means that with complement as limiting agent (guinea pig C') the pace-making step is a reaction not requiring a divalent cation. Hence, the hypothesis can be constructed that the Ca^{++} and Mg^{++} steps are completed within the first few minutes of reaction, and that thereafter comes a process not involving either of these divalent cations, and that consequently EDTA is without effect, unless added during the early stages of reaction. This hypothesis implies that there is a pile-up of the intermediate product formed in the reactions requiring Ca^{++} and Mg^{++} .

In line with this reasoning it has been

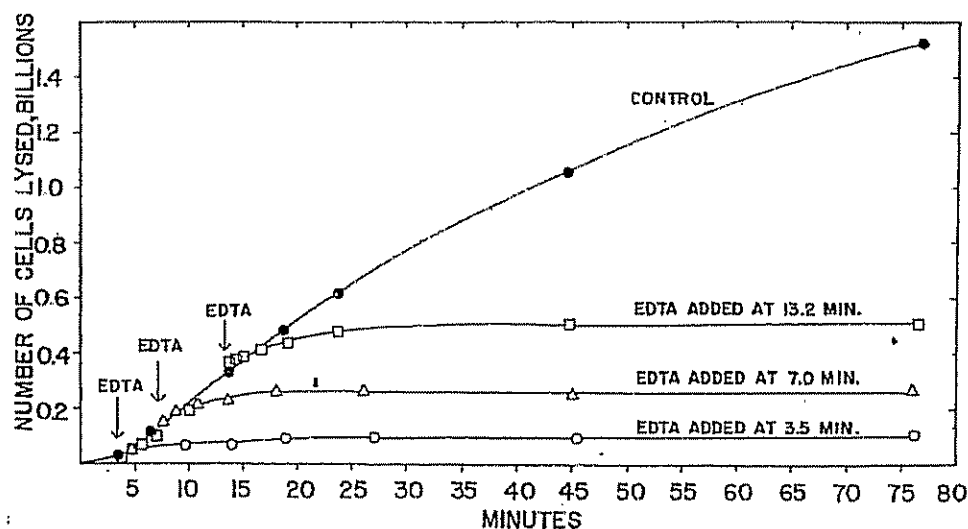


FIG. 52. The limited antibody system can be stopped at any time by addition of EDTA (from ref. 267).

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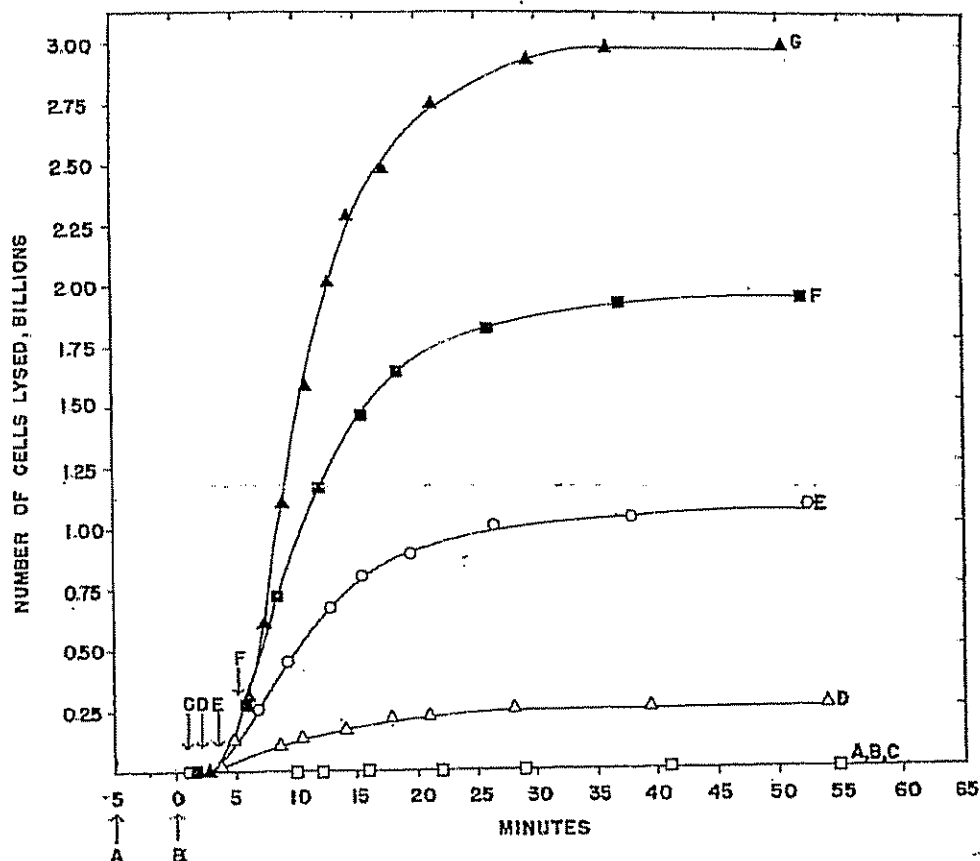


FIG. 53. Addition of EDTA to a limited complement system does not stop hemolysis, unless added during the first few minutes. Reaction mixtures A and B received EDTA before complement. EDTA was added to C, D, E and F at 1.0, 2.1, 3.7 and 5.2 minutes after complement addition; respectively, as indicated by arrows. Curve G represents the course of hemolysis in a control reaction mixture which did not receive EDTA (from ref. 267).

found that brief treatment of EAC'1, 4 with undiluted complement in the cold, and in the presence of Mg^{++} , leads to the formation of an intermediate product, which will hemolyze on treatment with C'y, or with whole complement, in the presence of EDTA. By means of tests with R1, R2, R3 and R4 it was shown that this new intermediate product comprises components C'1, C'4 and C'2 (133). Furthermore, it was found that the component of complement which acts on this product in the presence of EDTA, i.e., absence of divalent cations, is C'3. Subsequent studies showed

that the preparation of EAC'1, 4, 2 can be accomplished in a single operation by treatment of EA with undiluted complement, in the presence of both Ca^{++} and Mg^{++} , at $0^{\circ}C$. for about fifteen minutes. After this, the cells are washed with cold buffer, stored at $0^{\circ}C$. for four hours, in order to eliminate any E^* , as explained below, and after another cold washing they are ready for use.

Another approach leading to the recognition and isolation of EAC'1, 4, 2 emerged from studies by Leon, Plescia and Heidelberger (106) on the properties of two frac-

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tions of pig complement, one comprising C'1, C'3 and C'4, and the other containing C'1 and C'3 (cf. earlier studies by Jonsen, Manski and Heidelberger, ref. 143). Both of the fractions appear to lack C'2, as ascertained by tests of the classical type with R2, and therefore do not hemolyze EA. However, they produced hemolysis when added to EA previously treated at 37° C. with a sublytic dose of guinea pig complement, in the presence of Ca^{++} and Mg^{++} (106). Furthermore, the lytic effect of the C'1, 3 fraction on such treated cells was maintained almost undiminished after heating at 56°C. for one hour. From this, as well as from related observations obtained in this laboratory (45), Leon *et al.* concluded that treatment of EA with a sublytic dose of guinea pig complement presumably leads to formation of EAC'1, 4, 2.

Parenthetically it might be pointed out that Pillemer *et al.* (118) and Silverstein (144), have studied the reactivity of so-called "persensitized" cells, which are prepared in a similar fashion, and which in terms of present knowledge are more or less equivalent to cells in the state EAC'1, 4, 2, although the presence of E* (see below) as contaminant in these preparations cannot be excluded on the basis of available information.

From these findings it became possible to describe the reaction series of sensitized erythrocytes, EA, with complement as follows: First, EA reacts with C'1 in the presence of Ca^{++} , resulting in formation of an intermediate product designated EAC'1 which then reacts with C'4 to yield EAC'1, 4. This product then reacts with C'2 in the presence of Mg^{++} , yielding another intermediate product, EAC'1, 4, 2. In turn, this reacts with C'3 without requirement for a divalent cation. The product of this reaction can be designated by the symbol EAC'1, 4, 2, 3. These results are essentially in accord with the deductions of Pillemer *et al.* (118) and of Bier and Trapp (119). A cell in the condition EAC'1, 4, 2, 3 is damaged and will

lyse spontaneously in a period of time, the length of which depends on temperature and the amount of C' used for treatment.

The Terminal Transformation Reaction

The action of C'3 on EAC'1, 4, 2, the last step of the complement reaction sequence, does not produce immediate lysis, but some kind of intrinsic cellular change, requiring appreciable time, takes place before the lytic event. This terminal transformation reaction was discovered in experiments in which sensitized erythrocytes were treated with complement in the presence of both Ca^{++} and Mg^{++} at 37°C. for only about 4 minutes. At the end of this brief period, the reaction was interrupted by sudden chilling and immediate centrifugation. It was found that the sedimented cells continue to hemolyze after removal of the fluid phase, and even after several washings (cf. Fig. 54). Obviously, the cells were in a damaged condition, designated E*, which led to lysis without action of any complement factor in the fluid phase of the reaction system. Since cells in the condition E* could be isolated as an intermediate product from a reaction system run for a short period of time at 37°C. in the presence of both Ca^{++} and Mg^{++} , it was evident that the transformation of E* to ghosts is a relatively slow reaction. Kinetic experiments (145), such as that shown in Figure 55, indicated that complete transformation of a population of cells in the state E* into ghosts requires about forty minutes at 37°C. and approximately four hours at 0° C. Furthermore, it was found that the extent of reaction, i.e., the final degree of lysis, was not influenced appreciably by changes of temperature, moderate variation (i.e., from 0.143 M NaCl to 0.19 M NaCl) in ionic strength of the diluent, and was unaffected by the presence of a chelating agent in moderate concentration (e.g., up to 0.01 M EDTA).

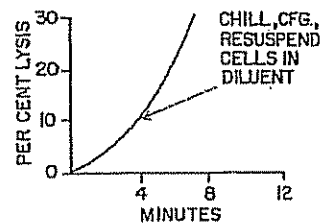
In view of the spontaneous nature of the terminal transformation reaction, it

Complement and Complement Fixation

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PERCENT LYSIS	64	62	61	59	48	62

FIG. 54. Schematic drawing of a kinetic hemolysis curve of the reaction between EA and C', showing the time at which the process was interrupted by sudden chilling and centrifugation. The sedimented cells were resuspended in buffer, and portions of the suspension were added to a series of tubes containing different quantities of diluent. Following incubation, the degree of lysis was measured. As shown in the tabulation, the extent of dilution did not affect degree of lysis appreciably. The portion of cells kept at 0°C. for one and one-half hours did not achieve the same degree of lysis, but in other experiments it was found that after storage at 0°C. for four hours the same extent of lysis was achieved as at 37°C. Furthermore, washing of the cells followed by resuspension in fresh diluent and incubation, did not alter the final degree of hemolysis.

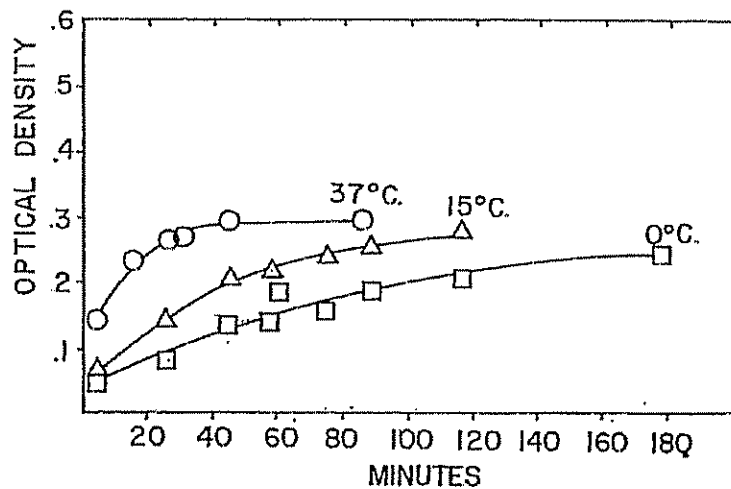


FIG. 55. Kinetics of the terminal transformation reaction (conversion of E* to ghosts) at different temperatures. The origin of the coordinate system is the time at which the resuspended cells were added to warm or cold diluent to yield mixtures at the desired temperatures. The curves do not go through the origin because some lysis took place at the time of centrifugal separation and the admixture of the resuspended cells with the warm or cold buffer (from ref. 145).

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would be expected to follow first-order kinetics. A study by Hoffmann *et al.* (146) has revealed slight, but significant deviation from first-order behavior, but most of this could be explained by technical factors. It was also found that the magnitude of the rate constant of the terminal transformation step depends on the source of the complement. Guinea pig serum yielded the highest rate, monkey serum gave a considerably lower value, and that for human serum was smallest. As a consequence, the pile-up of cells in the state E^* is much greater in hemolytic reactions with monkey or human than with guinea pig complement. This is of significance in kinetic analyses, since the speed of lysis does not reflect the velocity of complement action if the E^* pile-up is large. For example, as shown in Figure 56, the speed of E^* formation with monkey complement is much greater than the velocity of lysis (ghost formation).

Information on the nature of E^* has come from recent studies with Krebs ascites tumor cells. On exposure to antibody and complement these cells exhibit cytoplasmic swelling, rapid loss of intracellular K^+ , amino acids and ribonucleotides, and

a less rapid loss of cell protein and ribonucleic acid (147, 148). At the same time, the cells become permeable to the Na^+ of the medium. These changes can be explained by assuming production of "holes" in the cell membrane large enough to permit rapid exchange of inorganic cations and small molecules, but not of macromolecules. The resulting disturbance of osmotic regulation leads to swelling, and consequently, macromolecules become able to pass the cell membrane. If protein is added to the medium in concentration sufficient to balance the colloid osmotic pressure of the cells, swelling is prevented. Under these conditions, the cells lose K^+ and take up Na^+ , but macromolecules are not lost from the cells (149). Similar experiments with erythrocytes (149) suggest that E^* is a cell which has suffered impairment of osmotic regulation and that the terminal transformation reaction is a process of osmotic lysis (cf. 150).

Decay of $EAC'1, 4, 2$

The intermediate product $EAC'1, 4, 2$ is unstable, i.e., it loses its ability to react with $C'3$ (45). Its half-life is about eight

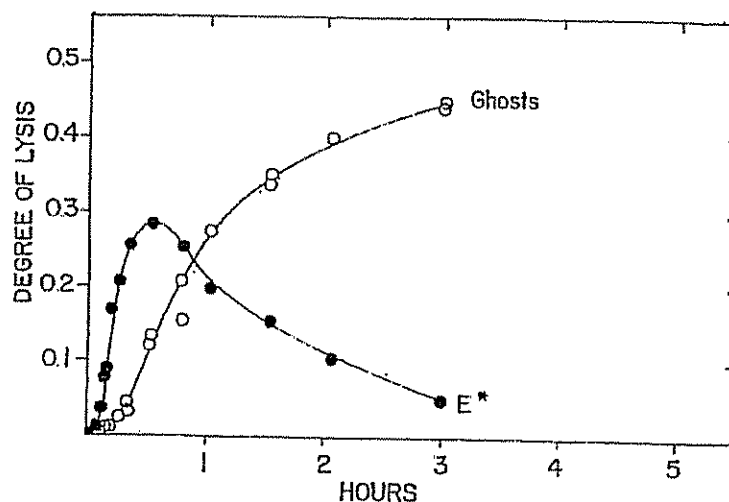


FIG. 56. Kinetic analysis with monkey complement by the ghost and E^* sampling techniques.

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minutes at 37°C., about twenty minutes at 30°C., and about ten hours at 0°C. The rate of decay is not affected by EDTA. Decayed EAC'1, 4, 2 can be lysed with C'y, or with purified C'2, followed by EDTA-complement, as a source of C'3. This means that the decayed cells are in the state EAC'1, 4.

In order to explain the significance of the decay of EAC'1, 4, 2 it will be necessary to consider the process of immune hemolysis in terms of the molecular reactions taking place upon the cell surface. There are approximately 5000 Forssman combining sites, S, on the sheep erythrocyte, as determined by measurements of the maximal capacity to bind Forssman antibody. If 1000 molecules of Forssman antibody are used, which is the usual amount for optimal sensitization in the titration of guinea pig complement, and if we accept the bimolecular sensitization hypothesis of Weinrach and Talmage (60), there should be about 125 sensitized sites, SA₂, per cell, assuming negligible transfer of antibody. On treatment with whole guinea pig complement at 0°C., in the presence of Ca⁺⁺, about 50-90 SA₂C'1, 4 per cell can be generated (the method for estimating the number of SA₂C'1, 4 per cell will be explained below), and in presence of Mg⁺⁺, these sites react with C'2 to form SA₂C'1, 4, 2, the extent of conversion depending on the quantity of C'2. However, SA₂C'1, 4, 2 sites are unstable, and as shown by Borsos *et al.* (68), they revert to SA₂C'1, 4 at a rate which depends upon temperature. This reversion is not a simple dissociation of C'2, since active C'2 is not released into the fluid phase. It was also shown in (68) that SA₂C'1, 4, 2 sites which have decayed to SA₂C'1, 4 can be restored to the state SA₂C'1, 4, 2 by treatment with purified C'2, and that such decay and regeneration can be performed repeatedly without loss of reactivity (68a).

Further discussion of this problem requires introduction of the one-site concept

of immune hemolysis. There are compelling reasons, to be presented later, for assuming that production of a *single* lesion in the erythrocyte membrane will lead to lysis, i.e., a cell in the state E* has at least one such lesion, designated by the symbol S*. From the work of Green *et al.* (149), S* might be considered a "hole" which permits exchange of Na⁺ and K⁺, as well as small molecules. Thus, the production of one S* upsets the osmotic equilibrium of the cell, which leads to swelling and eventual lysis.

Due to the fact that C'3 acts relatively slowly, the decay of SA₂C'1, 4, 2 imposes a significant interfering process which limits the efficiency of production of S*, i.e., due to the decay, only part of the available SA₂C'1, 4 can be converted to S*, the extent of conversion being dependent on the supply of C'2 and the concentration of C'3. If a very large amount of C'2 is available, i.e., if there is a large excess of C'2 over SA₂C'1, 4, the sites will be maintained in the state SA₂C'1, 4, 2 for a long time, through continuous cycling, and as a consequence, in the presence of C'3, a high proportion can be converted to S*. Conversely, if C'3 is present in high concentration and C'2 is available in moderate excess over SA₂C'1, 4, a high degree of conversion can be expected. However, if neither C'2 nor C'3 is present in high concentration, only a small proportion of SA₂C'1, 4 will go to S*. This is the situation in the titration of guinea pig complement, where, under the usual experimental conditions, only about 1 or 2 per cent of SA₂C'1, 4 become S*. On the other hand, in the titration of hemolytic antibody, in which a high concentration of complement is used, the efficiency of conversion is much higher.

In certain quantitative measurements of complement and its components, it is necessary to know the precise rate constant of decay of SA₂C'1, 4, 2 at a given temperature. Experimental measurements of this constant are performed by pipetting

portions of an ice-cold suspension of EAC'1, 4, 2 into a series of test tubes in an ice-bath. The entire rack of tubes is then transferred to a water bath at the desired temperature, say 30°C. or 37°C., and when the temperature of the contents of the tubes reaches that of the water bath, a time clock is started. Pre-warmed C'y (or whole C'), containing 0.005 M EDTA, is then added to the individual tubes at suitable intervals of time. In this manner, EAC'1, 4, 2 is permitted to decay for varying periods of time prior to initiation of reaction with C'3. Following addition of C'3, a period of about ninety minutes is allowed for the lytic process to reach an endpoint. The degree of lysis in each of the tubes is then determined by photometric analysis of the fluid phase for oxyhemoglobin.

The data so obtained furnish a relation between the proportion of cells which are lysable, y , and the time of incubation of EAC'1, 4, 2. However, since the decay is considered to be a molecular process, what we desire to know is the relation between the number of SA₂C'1, 4, 2 and the time of incubation.

Since one S* is believed to be necessary and sufficient for lysis of a cell, it follows from the Poisson distribution that the negative logarithm of the surviving cells should equal the average number of S* per cell, which is designated by the symbol z , i.e.,

$$z = -\ln(1-y)$$

Next, we must inquire into the relation between z and the number of SA₂C'1, 4, 2 per cell, which are present at the moment of sampling. This raises the question of the efficiency of conversion of SA₂C'1, 4, 2 to S*. As pointed out above, with a large concentration of C'3, the efficiency should be high; experimentally, it has been found feasible to attain better than 90% conversion. Therefore, under these conditions, z should be approximately equal to the average number of SA₂C'1, 4, 2 per cell at the moment of C'3 addition.

Experiments conducted with a low concentration of C'3 pose a more difficult problem since under these conditions, only a small proportion of the SA₂C'1, 4, 2 present at the moment of C'3 addition will go to S*. The magnitude of this proportion can be evaluated from the equations describing the action of C'3, as shown in the theoretical section. However, it is not necessary to do this, since the theoretical analysis of the C'3 reaction indicates that the ratio between z and the average number of SA₂C'1, 4, 2 per cell should be dependent only on the concentration of C'3 (this will be the case only if experimental conditions are chosen so that consumption of C'3 is negligible). This means that z can be considered to be directly proportional to the average number of SA₂C'1, 4, 2 per cell. For this reason, and since the decay is believed to follow first-order kinetics, it is considered valid to evaluate the decay data by plotting the logarithm of z vs. time.

A typical decay experiment at 37°C., shown in Figure 57, indicates a half-life of about eight minutes, which corresponds to a rate constant of about 0.09 min.⁻¹. At 30°C. the rate constant equals 0.030 min.⁻¹, and at 0°C. it is approximately 0.001 min.⁻¹.

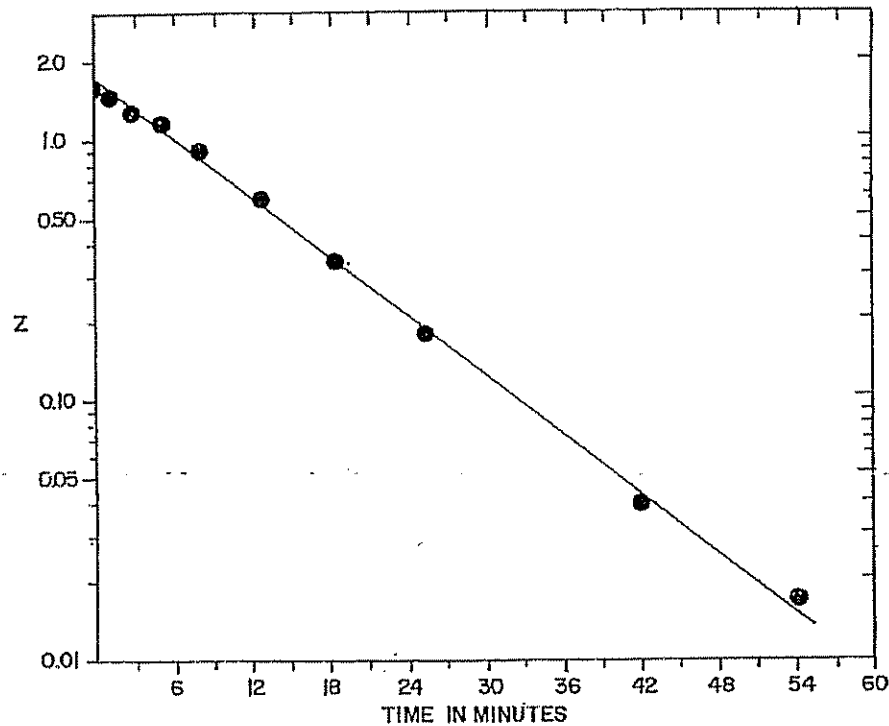
These values represent the best estimates available at present. Deviations of 10 or 20%, and sometimes even larger, have been encountered, possibly due to non-Poissonian distribution of C'2 upon the cells or consumption of C'3. This problem is still under active investigation.

From decay experiments it is possible to evaluate the number of SA₂C'1, 4 per cell. Cells in the state EAC'1, 4 are treated with C'2 in excess so as to convert all sites to SA₂C'1, 4, 2, followed by washing in the cold to remove excess C'2. The cells are then warmed quickly to the desired decay temperature. Samples taken at suitable intervals are treated with C'3 in high concentration so as to lyse practically all cells with at least one SA₂C'1, 4, 2. The

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FIG. 57. Kinetics of the decay of EAC'1, 4, 2 at 37°C. $z = -\ln(1-y)$.

natural logarithm of z , calculated from y , as indicated above, is plotted vs. time and from the line so obtained the value of z at zero-time is estimated. Since an excess of C'2 and a high concentration of C'3 were used, this should approximately equal the number of SA₂C'1, 4 per cell.

The instability of EAC'1, 4, 2 plays an important role in the hemolytic titration of complement because the action of C'3 is quite slow, and consequently, the intermediate EAC'1, 4, 2 accumulates. C'2 is used up quickly, and thereafter, reaction of EAC'1, 4, 2 with C'3 proceeds in competition with its decay. This competition serves to explain the "volume" or concentration effect observed in the titration of complement (cf. section on the factors influencing the hemolytic activity of complement). Since the rate of decay is not influenced by the concentration of react-

ants, while the reaction rate of C'3 increases with concentration, the formation of E*, and hence the degree of lysis, is favored by increasing the concentration of reactants.

Kinetics of Formation of EAC'1, 4, 2 from EAC'1, 4 by Action of C'2

It has been shown by Borsos *et al.* (68) that formation of EAC'1, 4, 2 from EAC'1, 4 starts immediately on addition of purified C'2, i.e., there is no lag. In a typical experiment, shown in Figure 58, peak activity was reached in about five minutes at 37°C., while about thirteen minutes were required at 30°C. The absence of an induction period is interpreted to mean that formation of one SA₂C'1, 4, 2 on a cell makes that cell susceptible to action of C'3, i.e., one effective hit by C'2 on a cell in the state EAC'1, 4 transforms

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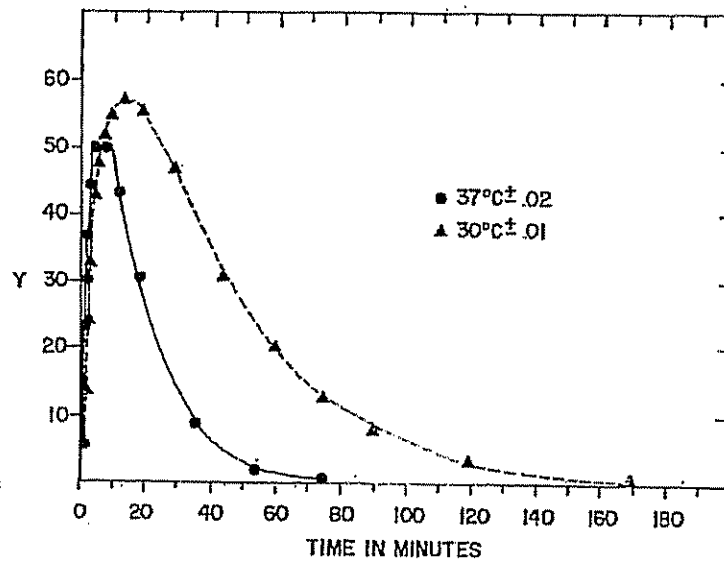


FIG. 58. Reaction between EAC'1, 4 and C'2 measured in terms of the concentration of EAC'1, 4, 2 (Y) as a function of time.

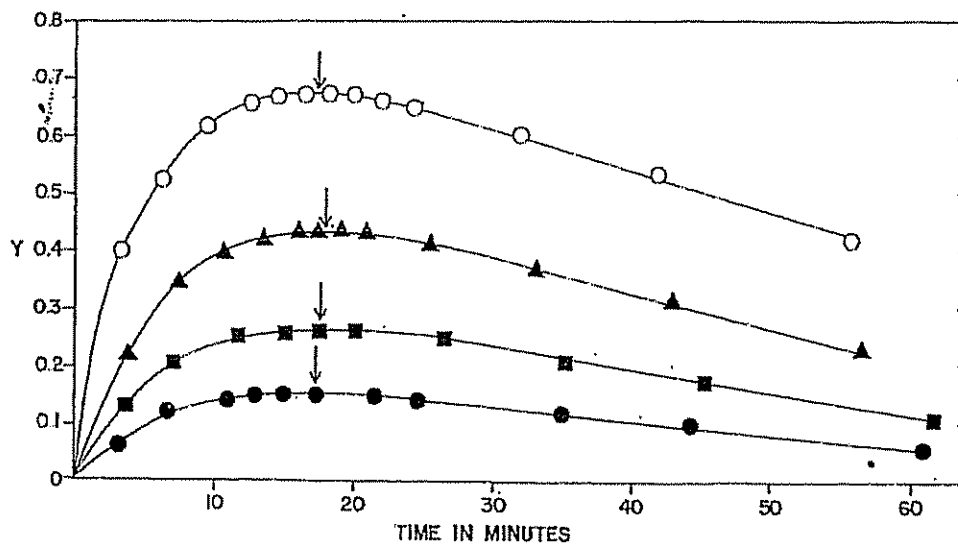


FIG. 59. Kinetics of EAC'1, 4, 2 formation with different concentrations of C'2 and constant EAC'1, 4. (30°C).

—○— C'2 dilution 1/1000; —▲— C'2 dilution 1/2000;
—■— C'2 dilution 1/4000; —●— C'2 dilution 1/8000.

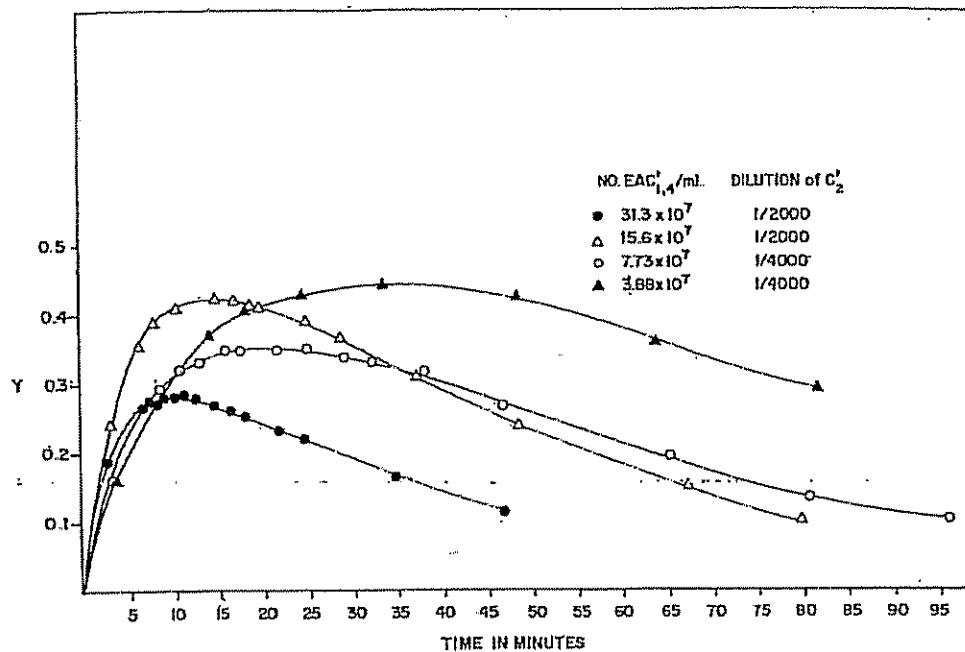


FIG. 60. Kinetics of EAC'1, 4, 2 formation with constant C'2 and varying concentration of EAC'1, 4. (30°C.)

that cell to the state EAC'1, 4, 2. It follows that reaction of one SA₂C'1, 4, 2 with C'3 transforms EAC'1, 4, 2 to E*, i.e., a single damaged site, S*, on a cell is believed to be sufficient for eventual lysis of that cell.

A theoretical analysis of the kinetics of SA₂C'1, 4, 2 formation will be given later. From the resulting equations it can be predicted that the peak-time of SA₂C'1, 4, 2 formation is independent of C'2 concentration. As shown in Figure 59, this has been verified experimentally. Furthermore, as expected from theory, the peak-time varies inversely with the concentration of EAC'1, 4, as shown in Figure 60. This is equivalent to variation of SA₂C'1, 4 per cell.

C'2 can be titrated by a two-step procedure: (1) Reaction of EAC'1, 4 with C'2 at 30°C for t_{max} minutes, where t_{max} is the peak time applicable to the EAC'1, 4 preparation being used. (2) Lysis of the EAC'1, 4, 2 so formed by C'3 in high con-

centration (EDTA-C'). The titration curve so obtained, plotting y , the degree of lysis, as a function of relative C'2 concentration, as shown in Figure 61, is not sigmoidal, and this supports the one-hit

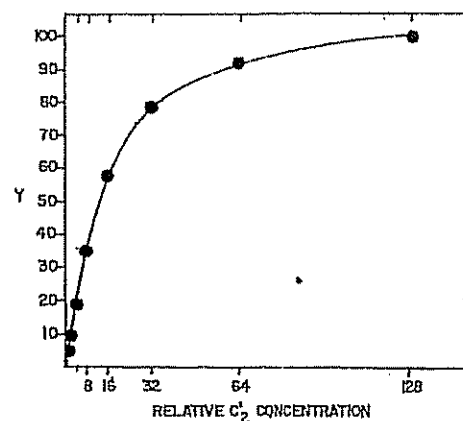


FIG. 61. Maximal extent of EAC'1, 4, 2 formation from EAC'1, 4, plotted as a function of the relative concentration of C'2 (30°C.).

concept. It follows from this concept (as explained above) that a plot of $-\ln(1-y)$ vs. $C'2$ concentration should yield a straight line. As shown in Figure 62, this holds true up to about 60 per cent lysis (the small deviation beyond 60 per cent may be due to consumption of $SA_2C'1, 4$ and $C'3$). From the amount of $C'2$ yielding $z = 1$, which corresponds to 63% lysis, it is possible to calculate the *absolute* molecular concentration of $C'2$ by application of the appropriate correction factor for the extent of decay prior to addition of $C'3$. For details of calculation, see experimental procedures.

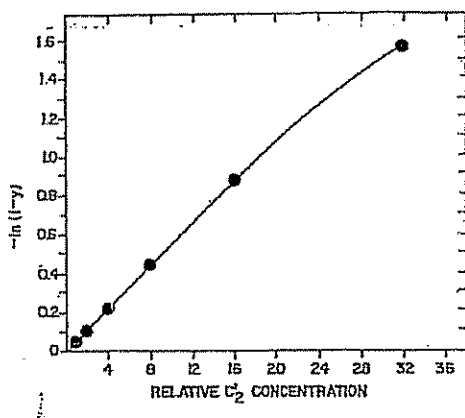


FIG. 62. Maximal extent of $SA_2C'1, 4, 2$ formation from $SA_2C'1, 4$ plotted as a function of the relative concentration of $C'2$; $-\ln(1-y)$ indicates the average number of $SA_2C'1, 4, 2$ per cell.

Kinetics of Formation of $EAC'1, 4$ from $EAC'1$ by Action of $C'4$

Initial studies by Hoffmann (140) indicate that formation of $EAC'1, 4$ proceeds without lag, following addition of chromatographically purified $C'4$ to $EAC'1$. This means that one $SA_2C'1, 4$ is sufficient to render a cell susceptible to lysis by $C'2$ and $C'3$, furnishing independent confirmation of the one-site theory of lysis. Furthermore, plots of z vs. concentration of $C'4$ have been found to be linear up to 99% lysis, in agreement with the one-site theory. Measurements of $C'4$ on an *absolute* molec-

ular basis are performed in a manner analogous to those of $C'2$, except that no correction for decay is needed, since a large excess of $C'2$ and a high concentration of $C'3$ are used to effect practically complete conversion of $SA_2C'1, 4$ to S^* . On the basis of such measurements, it is possible to make $EAC'1, 4$ containing a known number of $SA_2C'1, 4$ per cell (i.e., by reacting $EAC'1$ with a known number of $C'4$ molecules). The $SA_2C'1, 4$ content so calculated should agree with that determined by the decay method.

The Dual Nature of $C'3$

The conversion of $EAC'1, 4, 2$ to E^* by action of $C'3$ and the competing decay of $EAC'1, 4, 2$ have been studied in detail by Rapp (151). He found that the percentage of lysis varies in a sigmoidal fashion with the concentration of $C'3$ and that it is independent of the cell concentration, provided consumption of $C'3$ is negligible, which is the case under the usual experimental conditions. Furthermore, it was found that the initial velocity of E^* formation is dependent on the square of the $C'3$ concentration, which suggests that two molecules of $C'3$ are involved. In accord with this, Rapp found that kinetic curves of the formation of E^* from $EAC'1, 4, 2$ by action of $C'3$ exhibit a lag (90). Initial experiments (151, 23, 7) had failed to show this lag due to faulty sampling techniques, and this had led to the erroneous idea that two molecules of $C'3$ react simultaneously, perhaps as a dimer in equilibrium with a monomeric form of $C'3$. This error was uncovered through theoretical studies of the reaction mechanism which indicated the need for improvement in the sampling procedure with respect to the efficacy of stopping the action of $C'3$ on withdrawal of samples. By use of a revised procedure, Rapp found a slight lag (90), as shown in Figure 63. This indicates that formation of E^* from $EAC'1, 4, 2$ requires at least two successive steps.

Complement and Complement Fixation

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Through chemical fractionation of guinea C'3a and C'3b, both of which are required for transformation of EAC'1, 4, 2 into E*. pig serum Rapp (90) then succeeded in separating two distinct factors, designated Recently, an improved separation of these

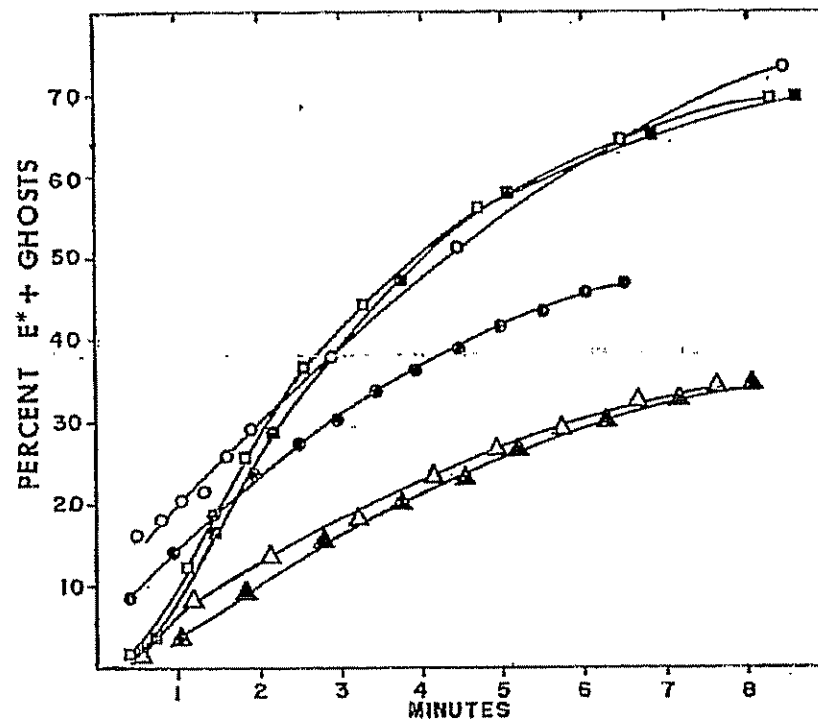


FIG. 63. Kinetics of E* formation from EAC'1, 4, 2. In the description that follows, the term *new sampling method* refers to the use of the improved sampling technique in which the kinetic experiment is performed in a cold room at about 5°C. instead of room temperature, samples are centrifuged immediately in cold (5°C.) table centrifuges rather than at room temperature, supernatant fluids are poured off immediately after centrifugation, and the unused cells are resuspended in fresh buffer before further incubation at 37°C., instead of incubating the sedimented cells in the presence of the original supernatant fluid.

Open circles: old sampling method..... C'3 1/100.
Solid circles: old sampling method..... C'3 1/115.
Open squares: new sampling method, 1.0 ml. of sample + 5.0 ml. of stop diluent..... C'3 1/60.
Solid squares: new sampling method, 1.0 ml. of sample + 10.0 ml. of stop diluent..... C'3 1/60.
Open triangles: new sampling method, 1.5 ml. of sample + 3.0 ml. of stop diluent..... C'3 1/90.
Solid triangles: new sampling method, 1.0 ml. of sample + 5.0 ml. of stop diluent..... C'3 1/90.

The reaction mixtures contained 3×10^7 erythrocytes per ml. and 5.0 ml. of the C'3 dilutions indicated for each experiment in a total volume of 25.0 ml. It should be noted that quantitative comparisons among different experiments can be made only if EAC'1, 4, 2 of the same reactivity is used (i.e., with the same number of SA₂C'1, 4, 2 per cell). This is the case for the open and solid squares, and for the open and solid triangles, respectively.

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factors has been achieved by chromatographic fractionation of guinea pig serum on DEAE-cellulose (152).

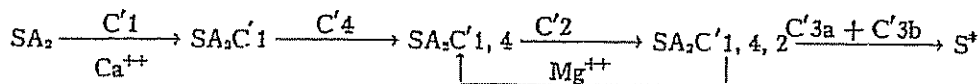
According to Taylor and Leon (153), who separated the corresponding factors from human serum, C'3a reacts with EAC'1, 4, 2 to form an intermediate product, EAC'1, 4, 2, 3a which then reacts with C'3b to form E*. With guinea pig complement, Rapp has obtained data indicating that C'3b reacts before C'3a. However, the intermediate product appears to be unstable on washing, and therefore it has not yet been possible to study the reactions of C'3a and C'3b as separate steps. More work is required to resolve this difference.

Adequate studies of the extent of removal of these factors from guinea pig serum by zymosan are not yet available. For lack of this information, there is uncertainty as to the precise nature of R3, and therefore it is difficult to interpret studies based on the use of this reagent, such as, for example, Arday's work on the purification of C'3 (154).

Recent unpublished experiments by Rapp indicate that C'3 comprises three distinct factors.

Summary

The action of guinea pig complement, in terms of the molecular reactions at the cell surface, may be summarized as follows:



Under the conditions usually prevailing in the titration of guinea pig complement, i.e., optimal sensitization with antibody, as well as optimal Ca^{++} and Mg^{++} , the action of C'1, C'4 and C'2 takes place within the first few minutes leading to accumulation of many $SA_2C'1, 4, 2$ on each cell (probably 50 to 100). Reaction of one of these with C'3 transforms a cell to the state E*. The action of C'3 is relatively slow (al-

though detailed information is not yet available, it appears likely that the C'3b reaction is the slow one) and hence, the extent of E* formation depends on the outcome of the competition between the action of C'3 (presumably C'3b) and the decay.

What then is the meaning of the over-all complement titer? This question is of importance in the interpretation of the changes in complement titer which occur in certain diseases, or following certain experimental treatments of animals. In former days, it was believed that the over-all complement titer is determined by the component present in lowest titer, the "limiting component" concept of Hegedus and Greiner (99), but in light of present day knowledge, such a simple interpretation is no longer tenable. The key to this question lies in the action of C'3 on EAC'1, 4, 2, and the decay of this intermediate. It has been pointed out that in the presence of a high concentration of C'3 the decay will not play a significant role, because most $SA_2C'1, 4, 2$ would be converted to S^* under these conditions. Alternatively, if a very large amount of C'2 is available, the decay will not interfere, because of continuous cycling. However, the concentrations of C'2 and C'3 in guinea pig serum are not sufficiently great to attain these conditions, and therefore, the over-all titer of guinea pig com-

plement is dependent upon both of these components, i.e., changes in either C'2 or C'3 will influence the over-all titer. (Too little information is available on the mechanism of action of C'3a and C'3b to permit extension of the present discussion with respect to these factors.)

What about C'1 and C'4? Here too, information is a bit scarce, but certain general deductions can be made. From study

of the conversion of EAC'1, 4 to EAC'1, 4, 2 by C'2, it is known that the concentration of SA₂C'1, 4 influences the efficiency of action of C'2. Furthermore, C'1 and C'4 appear to react in a stoichiometric fashion. Hence, if a large amount of antibody is used for sensitization, the extent of formation of SA₂C'1, 4 will depend on the supply of C'1 and C'4. The term 'optimal sensitization', as used in the titration of complement, means that antibody is in slight excess over the supply of C'1 and C'4, and consequently in the usual complement titration, the quantities of these components should influence the over-all titer, though to a smaller extent than C'2 and C'3.

In the titration of antibody, the situation is quite different. Here, antibody limits the extent of SA₂C'1, 4 formation, while C'1 and C'4 are present in excess. What about the influence of C'2 and C'3 in antibody titrations? This question may

be posed more concisely by asking whether all SA₂C'1, 4 are converted to S* under the conditions usually prevailing in the titration of hemolysin. Though precise data are lacking, it is known that the conversion is incomplete, i.e., the concentrations of C'2 and C'3 usually available are not high enough to achieve 100% efficiency. It follows that the antibody titer will be influenced by changes in the concentration of both of these factors. It is also to be expected that complete conversion of SA₂C'1, 4 to S* could be achieved by addition of a large amount of purified C'2, and this would seem to be one way for performing a titration of hemolytic antibody in absolute terms. This is of importance with respect to the hypothesis of Weinrach and Talmage (60) that two antibody molecules are needed for sensitization of a cell surface site. Rigorous proof of this concept is lacking, since in their experiments, conversion of SA₂C'1, 4 to S* was probably incomplete.

THEORETICAL ANALYSIS

Numerous attempts have been made to develop theoretical models of the action of complement and to derive mathematical equations describing them. Probably, the earliest work is that of von Krogh (54) in 1916 who analyzed the sigmoidal relationship between percentage hemolysis and amount of complement in terms of an adsorption process. A statistical interpretation was introduced in 1919 by Brooks (155), who attributed the sigmoidal relationship to heterogeneity of the cells with respect to lytic susceptibility. This interpretation is related to the concept that lysis of a cell is due to accumulation of damage, i.e., lysis occurs when a critical number of damaged sites has been produced. The sigmoidal shape of *kinetic* hemolysis curves has also been attributed to heterogeneity (Ponder 150), but his analysis took no account of the sequential action of the complement components

which would produce a sigmoidal kinetic lysis curve, regardless of other factors.

The multiple-hit concept was stated most clearly by Alberty and Baldwin (156), who pointed out that if the lytic action is a multiple-hit phenomenon (i.e., due to cumulative damage) it is not necessary to assume inhomogeneity in order to explain the sigmoidal response curve, since the action of complement on any given cell would be subject to an element of chance. In the mathematical theory proposed by Alberty and Baldwin the fraction, y , of the cells lysed is given by the binomial equation

$$y = \sum_{x=0}^{m-1} \binom{m}{x} p^x (1-p)^{m-x} \quad [1]$$

in which m is the total number of substrate regions, S , per cell, x is the number of reacted substrate regions on any one cell, p is the fraction of the total number of